

Delivery of Antibiotics to the Eye Using a Positively Charged Polysaccharide as Vehicle

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ABSTRACT The positively charged polysaccharide chitosan is able to increase precorneal residence time of ophthalmic formulations containing active compounds when compared with simple aqueous solutions. The purpose of the study was to evaluate tear concentration of tobramycin and ofloxacin after topical application of chitosan-based formulations containing 0.3% wt/vol of antibiotic and to compare them with 2 commercial solutions: Tobrex® and Floxal®, respectively. The influence of the molecular weight, deacetylation degree, and concentration of 4 different samples of chitosan on pharmacokinetic parameters (area under the curve values [AUC_{eff}] and time of efficacy [t_{eff}]) of tobramycin and ofloxacin in tears was investigated over time. It was demonstrated that the 2 chitosan products of high molecular weight (1350 and 1930 kd) and low deacetylation degree (50%) significantly increased antibiotic availability when compared to the controls, with AUC_{eff} showing a 2- to 3-fold improvement. The time of efficacy of ofloxacin was significantly increased from about 25 minutes to 46 minutes by the chitosan of higher Mw (1930 kd) at a concentration of 0.5% wt/vol, whereas a similar performance was achieved by a chitosan of low Mw (580 kd) at a concentration of 1.5% wt/vol in the case of tobramycin.

KEYWORDS: Chitosan, hydrogel, ophthalmic application, antibiotic, pharmacokinetics.

INTRODUCTION

Some external pathogenic bacteria as well as bacteria present in the physiological conjunctival flora such as *S. epidermidis*, *S. aureus*, and *P. aeruginosa* are potential causes of external ocular infections, including bacterial keratitis¹. Current treatment of such infections is primarily based on the topical administration of appropriate anti-infective agents. For example, aminoglycosidic antibiotics such as

tobramycin are often prescribed to eradicate external ocular infections because of its marked activity against *P. aeruginosa*. However, the appearance of growing patterns of resistance has led to the use of new antibiotics such as ofloxacin^{1,2}, a fluoroquinolone, which has been shown in clinical trials to be as effective as tobramycin and to act more rapidly regarding the reduction and disappearance of pathological symptoms³.

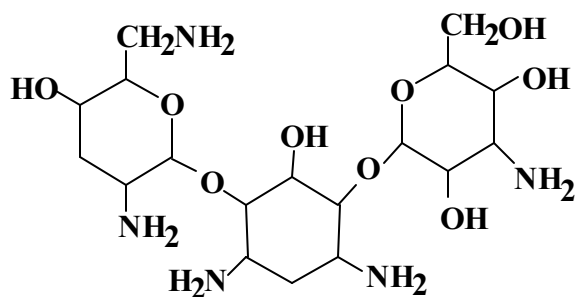
The success of an antibacterial therapy depends on features of the antibiotic, such as its antimicrobial spectrum, potency, and minimum inhibitory concentration (MIC^{90}) values, as well as on the ophthalmic vehicle. Indeed, an ophthalmic formulation that increases the contact time with the ocular surface should allow therapeutic levels to be maintained over a prolonged period of time. Chitosan was chosen as a vehicle to increase tobramycin and ofloxacin concentrations in ocular fluids because this biodegradable polysaccharide has been demonstrated to remain in the precorneal area significantly longer than a commercial solution⁴.

The aim of this study was to compare the performance of chitosan-based formulations with that of commercially available ophthalmic solutions, namely Tobrex® (Alcon, Fort Worth, TX) and Floxal® (Chauvin, Montpellier, France), and to determine whether increased corneal contact time due to the presence of a viscosifying and bioadhesive vehicle such as chitosan, as previously demonstrated⁴, can be correlated with prolonged concentrations of drugs in tears.

In addition, the molecular weight, deacetylation degree, concentration of the polysaccharide, as well as

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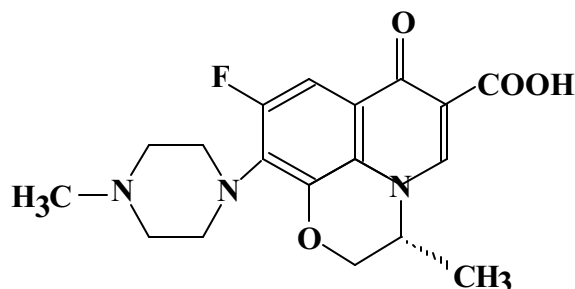
Figure 1. Chemical structures and physicochemical properties of tobramycin and ofloxacin.



Tobramycin

solubility in water (mg/mL): >500 (10)

pKa₁: 6.7
pKa₂: 8.3
pKa₃: 9.9 (11)



Ofloxacin

solubility in water (mg/mL): 3.23 (9)

pKa₁: 5.7
pKa₂: 7.9

the physicochemical characteristics of tobramycin and ofloxacin such as the constant of dissociation (pKa) and the lipophilicity, are discussed as parameters capable of influencing the duration of presence in tears of the bioactive substance above the required MIC⁹⁰ value.

MATERIALS AND METHODS

Materials

In the present study, we used the same types of chitosans as were previously used by Felt et al⁴. More precisely, 2 high purity grade chitosan salts of similar deacetylation degree (>80%) were purchased from Pronova Biopolymer (Oslo, Norway), namely chitosan hydrochloride UPCI 110 and chitosan glutamate UPG 210, which have the molecular weights of 160 kd and 580 kd, respectively. Two types of chitosan base (CHITO-1 and CHITO-2) of high molecular weight (1350 kd and 1930 kd, respectively) and low deacetylation degree (<60%) as well as tobramycin were kindly provided by Ciba Vision® (Duluth, GA). Ofloxacin was obtained from

Sigma (Buchs, Switzerland). Formulae and physicochemical characteristics of both antibiotics are shown in **Figure 1**.

Tobrex and Floxal were used as reference products, as both were commercially available. They contained tobramycin and ofloxacin, respectively, at a concentration of 0.3%.

Preparation of ophthalmic formulations

Each type of chitosan was dissolved at concentrations of 0.5%, 1.0%, and 1.5% wt/vol, at room temperature, under magnetic stirring in an isocryoscopic sterile phosphate buffer saline (PBS) solution, pH 7.4. Because higher concentrations of CHITO-1 and CHITO-2 lead to the formation of hydrogels of a viscosity too high to be easily and reproducibly applied to the eye, these types of chitosan were prepared only at the lowest concentration (0.5% wt/vol). The antibiotics (tobramycin or ofloxacin) were dissolved in the same medium as the polysaccharide at a concentration of 0.3% wt/vol. After complete dissolution of chitosan, solutions

containing the polysaccharide were poured into the preparation containing the antibiotic. Because tobramycin is basic when in solution, further adjustment with acetic acid (1% vol/vol in distilled water) to pH 6.0 to 6.2 was necessary to keep chitosan in solution.

Before administration onto the cornea, the final pH (pHmeter 691, Metrohm, Switzerland) and the cryoscopicity (automatic osmometer type Digital/L, Knauer, Germany) of all the formulations were checked, and each measurement was made in triplicate.

In vivo pharmacokinetic experiments

Male albinos New Zealand rabbits weighing approximately 4 to 5 kg and free of any ocular damage were used throughout the whole study as approved by the local ethics committees for animal experimentation.

A volume of 25 μL of the solution to be tested (formulations containing chitosan as well as commercial controls) was administered onto the cornea of the unanaesthetized animals using an adjustable micropipette (Assipettor-Digital®, Assistant, Germany). Tear samples were collected after 0, 1, 2, 4, 6, 8, 10, 15, 20, 30, 45, and 60 minutes after instillation, using 2.0 μL calibrated glass capillaries (microcaps Drummond®, Thomas Scientific™, Swedesboro, NJ). Samples were frozen at -25°C for further analysis of drug concentrations in tears. Each formulation was tested on 6 rabbits.

Determination of tobramycin concentration in tears

Concentrations of tobramycin in tears were determined using a fluorescent polarization immunoassay, which was previously used for the analysis of gentamicin^{5,6} on a TDx® analyzer (Abbott Laboratories, Dallas, TX). A commercially available kit of reagent (Abbott Laboratories) was used. The sensitivity of this method, which is defined as the minimal concentration distinguishable from the zero concentration with 95% confidence, has been determined by the supplier to be 0.18 $\mu\text{g/mL}$.

Determination of ofloxacin concentration in tears

Determination of ofloxacin in tears was carried out on a HP^{3D} CE system (Hewlett-Packard, Wilmington, DE), as described earlier⁷. The system consists of a capillary electrophoresis unit equipped with a diode array detector (DAD), an autosampler, and a high-

velocity air-cooled capillary cartridge. The HP^{3D} Chemstation software was used for instrument control, data acquisition, and data analysis. Hewlett-Packard capillaries with a 50 μm internal diameter (ID) (375 μm outside diameter [OD]) and 64.5 cm total length (56 cm from inlet to the detector window) were used for all experiments. These capillaries were made of fused silica and equipped with an extended path-length detection window of 150 μm ID ("bubble cell"). New capillaries were flushed for 3 minutes with 1 M NaOH, followed by 5 minutes with 0.1 M NaOH, and finally for 10 minutes with water. After each run, capillaries were flushed for 4 minutes with water, 2 minutes with 0.1 M sodium dodecyl sulphate, then 2 minutes with water, and finally 4 minutes with the separation buffer solution. The 100 mM PBS solution used for the separation was prepared as described in the Helvetic Pharmacopeia VII by dissolving 6.75 g of KH_2PO_4 in 500 mL of water to obtain a pH of 4.5. Preparation of tear samples was carried out as follows. A micro-vial (Hewlett-Packard, Waldbronn, Germany) was filled with 18 μL of water containing only 10% (vol/vol) of separation buffer to enhance sensitivity by stacking method and 10 $\mu\text{g mL}^{-1}$ of imipramine HCl used as an internal standard. Then the tear sample of 2 μL collected with the glass capillary was blown under a gentle nitrogen flow into the micro-vial. The vial was finally centrifuged for 5 minutes at 10 000 rpm (Avanti™ 30 Centrifuge, Beckman, Palo Alto, CA) before injection.

Samples (24 nL) were injected under pressure (5 kPa for 20 seconds), and electrophoresis was performed at a constant voltage of 20 kV (310 V cm^{-1}) after a 1 minute ramp step to avoid loss of sample at the injection⁸. The capillary was thermostatted at 25°C , and the detection was performed using the DAD (scanning from 190 nm to 600 nm). Electrophoregrams were monitored at 290 nm with a bandwidth of 3 nm for both ofloxacin and imipramine. To subtract the detector noise, the reference signal was fixed at 450 nm (bandwidth = 80 nm). In all experiments, areas were corrected by their respective migration times.

A typical electrophoregram is shown in **Figure 2**. Ofloxacin (migration time = 14.03) is completely separated from imipramine (migration time = 12.87) and tear constituents, which showed no absorption at 290 nm. The limits of detection and quantification were 0.5 $\mu\text{g mL}^{-1}$ (signal-to-noise ratio 3:1) and 2 μg

mL^{-1} (signal-to-noise ratio 10:1), respectively. The linearity of the response was demonstrated from 2 to $100 \mu\text{g mL}^{-1}$ with a correlation coefficient (r) of 0.9998.

Data analysis

The following parameters were calculated from the different time-concentration curves obtained after measuring the amount of antibiotic in tears: Area under the curve values (AUC_{eff}), which are considered representative of the availability of the active compound in the lachrymal fluid, were calculated using the trapezoidal rule. Performance of the different formulations containing chitosan was evaluated by calculating the AUC_{eff} ratio (ie, $\text{AUC}_{\text{eff chitosan formulation}} / \text{AUC}_{\text{eff collyrium}}$). The time of efficacy (t_{eff}), which in the case of an antibiotic can be defined as the time during which the concentration of active agent remains above its MIC^{90} value, was also determined. Comparison of these pharmacokinetic parameters between the different formulations tested and the commercial controls was achieved using a Student's t -test (unpaired samples), after ensuring that the data points followed a normal distribution.

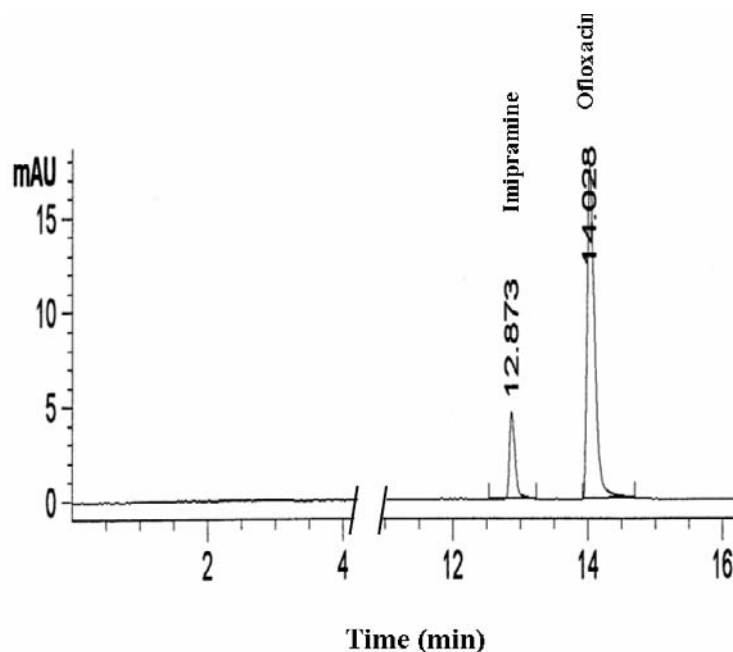


Figure 2. Separation of a sample containing imipramine (internal standard) and ofloxacin after administration of a solution of 0.3% ofloxacin in 0.5% UPCI 110 in the conjunctival sac.

RESULTS

Lachrymal availability of tobramycin and ofloxacin

Preparation of the formulations was simple and led to transparent viscosified solutions with characteristics allowing an ophthalmic administration, pH ranging from 5.8 to 6.3, osmolalities ranging from 280 to 315 mOsm/kg, and viscosities ranging from 10 to 477 mPa.s. It can be noted that CHITO-1 and CHITO-2, due to their relatively high Mw, took a long time to completely dissolve in PBS (approximately 2 days). Interestingly, as soon as the solutions containing chitosan were added to the suspensions of the poorly water-soluble ofloxacin, the antibiotic dissolved completely and rapidly. The solubility in aqueous media of fluoroquinolones is considerably increased by decreasing the pH from neutral to slightly acidic⁹. Indeed, Ross and Riley⁹ have determined that lowering the pH from 7 to 5 increased the solubility of ofloxacin from 3.23 mg/mL to 95.4 mg/mL. Therefore, it is proposed that this solubilization phenomenon is due to the fact that aqueous solutions of chitosan have a pH ranging from 5.5 to 6.5.

The AUC_{eff} of both antibiotics after topical administration of Tobrex and Floxal are presented in **Table 1**. It appears that the availabilities of tobramycin and ofloxacin in tear fluid were equivalent, having AUC_{eff} of approximately 3000 to 3500 $\mu\text{g/mL/min}$. This suggests that the characteristics (pK_a values, lipophilicity) of the active compounds do not exert any influence on this parameter.

In a previous study⁴, it was demonstrated by gamma scintigraphy that the presence of chitosan in an ophthalmic solution always resulted in a significant increase of the precorneal residence time when compared with commercial controls, regardless of the type of polysaccharide or of its concentration in the formulation. Hence, a corresponding increase of drug concentration in tears could be expected, based on the general hypothesis that prolonging precorneal residence time enables therapeutic drug levels to be maintained over longer periods of time. A general observation regarding tobramycin and ofloxacin is that the 2 types of chitosan of high Mw, CHITO-1 and CHITO-2, significantly improve antibiotic availability in tears, with the AUC_{eff} ratio showing a 2- to 3-fold improvement (**Table 1**). Only UPG 210 at 1.0% wt/vol was able to induce a similar significant

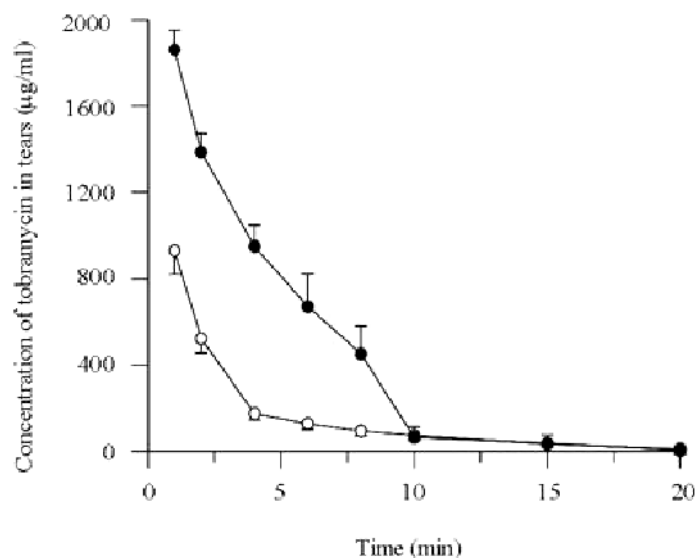
Table 1. Lachrymal Availability of Tobramycin and Ofloxacin After Instillation of 25 μ L of Various Formulations Based on Chitosan and Controls

Formulation	Viscosity (mPas) (4)	Tobramycin 0.3%		Ofloxacin 0.3%	
		AUC _{eff} ^a \pm SD (μ g/mL/min)	AUC ratio ^b	AUC _{eff} ^a \pm SD (μ g/mL/min)	AUC ratio ^b
Tobrex [®] (Alcon)	2.7	3321 \pm 2194	-	-	-
Floxal [®] (Chauvin)	3.0	-	-	3534 \pm 2373	-
UPCI 110 0.5%	10.0	5879 \pm 5554	1.77	2558 \pm 2298	0.72
1.0%	17.4	5362 \pm 3911	1.61	2285 \pm 348	0.65
1.5%	30.7	4604 \pm 2018	1.39	1746 \pm 605	0.49
UPG 210 0.5%	16.8	2953 \pm 2780	0.89	3393 \pm 1871	0.96
1.0%	54.5	6376 \pm 2249	1.91 ^c	3208 \pm 2285	0.91
1.5%	114.2	4980 \pm 2523	1.50	5306 \pm 2139	1.50
CHITO-1 0.5%	73.7	7485 \pm 2650	2.25 ^c	5812 \pm 2794	1.64 ^c
CHITO-2 0.5%	477.1	10722 \pm 2660	3.23 ^c	11543 \pm 4547	3.27 ^c

^aEfficacy area under the curve.^bAUC_{eff} chitosan formulation /AUC_{eff} collyrium.^cp < 0.05, Student's t-test, unpaired samples, comparison with controls.

improvement in the AUC_{eff} of tobramycin (AUC_{eff} ratio = 1.91, p < 0.05). In the case of tobramycin, the effect of CHITO-1 and, mostly, of CHITO-2 on the antibiotic availability was so marked that the difference with Tobrex was significant with a high degree of probability (p < 0.0005) especially considering the fact that the result came from an in vivo study (**Figure 3**).

The 2 other types of chitosan, UPCI 110 and UPG 210, did not show a significant influence on the AUC_{eff} (p > 0.05), but they did exert an opposite effect on tobramycin and ofloxacin. In fact, the presence of these types of chitosan at any concentration tended to increase the AUC_{eff} ratio of tobramycin (1.39 to 1.77), whereas it generally decreased that of ofloxacin (0.49 to 0.96). This finding was unexpected because tobramycin is highly hydrophilic and thus was expected to be rapidly delivered from chitosan solutions and eliminated by lachrymal drainage. This improvement may be explained by a physical entanglement between tobramycin and chitosan or by a chemical interaction perhaps due to hydrogen bonding, as both components possess numerous hydroxyl and amino groups.

**Figure 3. Tobramycin concentration profiles in tears (mean \pm SEM) after instillation of 25 μ L of (○) Tobrex[®] and (●) a formulation containing 0.5% CHITO-2.**

Time of efficacy of tobramycin and ofloxacin

The efficiency of the formulations was compared using t_{eff} , the time during which the antibiotic concentration remained above its MIC^{90} (ie, 4 $\mu\text{g/mL}$). Indeed, at this concentration both antibiotics are still efficient against sensitive bacterial strains such as *S. aureus*, *P. aeruginosa*, and *haemophilus sp*, which are commonly involved in external ocular diseases. However, it must be noted that for some strains, such as *S. pneumoniae*, ofloxacin is much more efficient, having a MIC^{90} of about 2 $\mu\text{g/mL}$ instead of the 16 $\mu\text{g/mL}$ of tobramycin.

As for availability, the t_{eff} of tobramycin and of ofloxacin after instillation of Tobrex and Floxal, respectively, were quite equivalent; both were about 20 minutes (**Figure 4**). This result is not in agreement with some other studies reporting that ofloxacin remained above its MIC^{90} value much longer than tobramycin. This can be easily explained by the fact that in previous studies, MIC^{90} values used for calculations were not considered to be equivalent¹, while in our study, they were considered to be equivalent. Indeed, Tang-Liu et al¹ found that ofloxacin remained above its MIC^{90} in human ocular tear fluid for 605 minutes versus 251 minutes for tobramycin, which is quite logical because MIC^{90} of ofloxacin against *S. aureus*, for example, was considered to be 0.5 $\mu\text{g/mL}$ versus 2.0 $\mu\text{g/mL}$ for tobramycin. Furthermore, Tang-Liu et al¹ obtained greater drug residence times than was the case in the present study, probably because of the numerous differences in experimental conditions, such as the volume and the regimen of instillation (single drop of 25 μL vs iterative administration of 1 drop), the sampling method (2 μL glass capillaries vs Schirmer's strips), and the species studied (rabbit vs human).

As shown in **Figure 4**, the time of efficacy of ofloxacin was significantly improved (1.8-fold increase, $p < 0.05$) by the presence of 0.5% wt/vol of CHITO-2. The same formulation did not show an identical effect on the hydrophilic antibiotic tobramycin. A similar improvement was achieved in the case of tobramycin by adding 1.5% wt/vol of chitosan UPG 210 (2.1-fold improvement, $p < 0.05$).

It is interesting to note that the influence of the different types of chitosan depended on the antibiotic present in the formulations. Indeed, the presence of chitosan induced, in most cases, a slight improvement

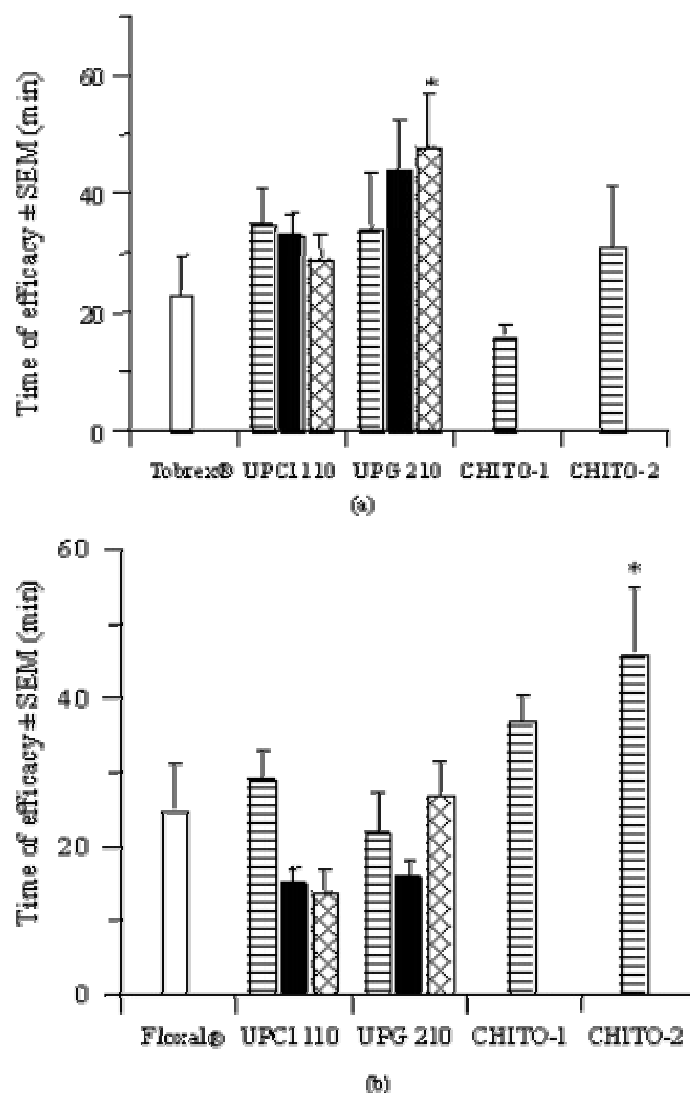


Figure 4. Time of efficacy \pm SEM (minutes) of tobramycin (a) and ofloxacin (b) after topical instillation of (□)controls and various formulations containing chitosan at (■) 0.5%, (■) 1.0%, and (▨) 1.5%.

in the t_{eff} of tobramycin, as was the case for AUC_{eff} , whereas only the chitosans of higher Mw (CHITO-1 and CHITO-2) were able to increase the parameters of ofloxacin; the other types of chitosan showed the opposite trend. Moreover, the t_{eff} of tobramycin seemed to be more influenced by the polymers of lower Mw (UPC1 110 and UPG 210), whereas the t_{eff} of ofloxacin was modified by CHITO-1 and CHITO-2 to a greater extent. According to this observation, we suggest that the mechanisms of interaction between chitosan and the antibiotics are different, being nonselective (eg, physical entanglement and/or hydrogen bonding) with tobramycin and selective (electrostatic) with ofloxacin. In fact, because

tobramycin is hydrophilic, it can be retained in the hydrophilic network of chitosan. In the case of ofloxacin, which exhibits hydrophobic features, an electrostatic interaction between its carboxylic group and the amino groups of chitosan may occur.

CONCLUSION

This study has demonstrated that although the evaluation of the ocular residence time using gamma scintigraphy is a very useful screening tool to identify promising formulations, it needs to be supplemented by direct measurement of drug levels in ocular fluid. Indeed, such experiments are necessary to show that increased duration of contact between the medication and the target site implies prolonged effective therapeutic drug levels. This study also showed that chitosans offer several advantages as ophthalmic carriers for ofloxacin. Indeed, the presence of chitosan promotes the solubilization of this antibacterial agent. Hence, it will avoid the need for additional components having the same role, such as surfactants, which are not always well tolerated by the eye. In addition, the highly viscous formulation based on the high molecular weight chitosan (1930 kd) has been shown to induce a 3-fold improvement of the availability and a 1.8-fold improvement of the t_{eff} of this fluoroquinolone. In most cases, an improvement of the AUC_{eff} and t_{eff} of tobramycin was achieved by using chitosan vehicles, independently of the Mw or the deacetylation degree values of the polysaccharide.

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